IN VIVO LOCAL DRUG DELIVERY OF NON-ELECTROACTIVE SPECIES

by

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Pressure ejection is the controlled expulsion of substances using applied air pressure from pulled multibarrel glass capillaries. The technique is frequently employed in neuroscience for the localized delivery of reagents to select brain regions of interest. Our lab has recently adapted a method to prevent unintentional leaking of reagent into the recording site. Here, those principles have been adapted to allow the local delivery of non-electroactive substances in vivo. A pressure-ejected system was coupled to fast scan cyclic voltammetry for real-time monitoring of ejections and a dye was introduced into the pipet barrels for visualization of ejection progress. This study shows that the dye had no effect on the electrically evoked dopamine response. Additionally, it shows the method proposed is applicable for fabrication and local employment of electroinactive reagents delivery of in vivo.

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1.0 INTRODUCTION

Dopamine (3,4-dihydroxyphenylethylamine), an important catecholamine neurotransmitter in the mammalian central nervous system (CNS), is of immense interest in the field of neuroscience. Numerous studies have established that dopamine plays significant roles in the regulation of a variety of behaviors, such as cognition, locomotion, and regulation of mood (Borland & Michael 2004; Michael & Borland 2007; Venton & Wightman 2003; Zhang et al 2007). Dysfunctions of the dopamine pathways are implicated in several neurological disorders including Parkinson's disease, schizophrenia, substance abuse, and attention deficit hyperactivity disorder (Borland & Michael 2004; Koob & Bloom 1988; Carlsson & Carlsson 1990; Venton & Wightman 2003; Zhang et al 2007). As a result, the dopaminergic system is a frequent target of pharmacological research in the CNS involving the development and investigation of therapeutic drugs (Taylor et al 2012).

Systemic administration (e.g. intravenous injection, intraperitoneal injection, oral gavage) of drugs for targeted action in the CNS is a popular mode of reagent delivery due to its convenience and simplicity. However, systemic drug delivery is non-specific and actively targets of all regions of the brain thereby confounding measured results. Moreover, drugs delivered systemically are subject to enzymatic degradation that may reduce their effects in the brain as well as diffusion barriers that might prevent their delivery to the site of action. Consequently, use of significantly high drug doses is sometimes required to ensure therapeutic drug levels are achieved in the brain and avoid erroneous conclusions regarding reagent efficacy (Herr et al 2010; Lalley 1999; Robinson et al 2008).

Local administration of drugs directly to the site of action restricts the actions and effects of drugs to a specific region of the brain, which simplifies the analysis and

circumvents the metabolic and diffusive barriers associated with systemic delivery. Local drug delivery / clearance is also faster and more controlled then systemic delivery (Herr et al 2010; Lalley 1999; Robinson et al 2008). In addition, it allows the use of much smaller amounts of reagent to reach therapeutically significant levels and bypasses the blood-brain barrier (Hanani 1997). Furthermore, local drug administration permits ultrastructural analysis of the action site, aids in the in vivo calibration of electrodes, and allows selective targeting of specific neuroanatomical structures such as individual neurons and distinct dopamine domains (Herr et al 2008; Moquin & Michael 2011; Moquin et al 2012; Peters et al 2004). Nevertheless, local delivery methods are more invasive than systemic ones and implanting the delivery devices and the delivery itself disrupts brain tissue potentially altering the neurochemistry of the tissue under investigation.

Over the years, research employing the focal application of reagents has contributed significantly to our understanding of the effects of drugs, both therapeutic and illicit, on the brain. Iontophoresis and pressure ejection have evolved as the primary means of locally delivering reagents in vivo due to their ability to quickly and selectively deliver small quantities of reagents to highly localized regions of the brain without significant disruption of ongoing neurochemical behavior (Herr et al 2008; Herr et al 2010; Lalley 1999; Moquin & Michael 2011; Moquin et al 2012).

The remainder of the chapter is divided into two main sections. The first introduces the methods of iontophoresis and pressure ejection, and includes a historical account on the development of each technique. It concludes with a summary of the advantages of using pressure ejection in conjunction with fast scan cyclic voltammetry (FSCV) at a carbon fiber microelectrode (CFE). The second part provides a brief summary of dopamine neurotransmission in the rat brain and introduces two techniques used to study it, microdialysis and FSCV.

1.1 METHODS OF LOCAL DRUG DELIVERY

Iontophoretic drug delivery involves the controlled ejection of charged substances into the microenvironment of the intact brain and relies on the movement of ions under the influence of an externally applied electrical current. In vivo iontophoretic delivery of reagents was first observed in 1936 by Sul et al who used it to eject acetylcholine intracisternally for the identification of cholinoceptive pressor regions in the brain stem (Lalley 1999). Development of iontophoresis, however, is generally credited to William Nastuk whose 1953 publication has been widely accepted as the first account of iontophoretic use. His paper describes the electrically controlled delivery of acetylcholine directly onto the neuromuscular junction using pulled capillaries (Herr et al 2010; Lalley 1999; Nastuk 1953). Two years later, del Castillo and Katz refined the technique, also using it to study the effects of acetylcholine on the neuromuscular junction (1955). In 1958 Curtis and Eccles became the first to use multi-barrel iontophoresis probes during their study of Renshaw cells (Herr et al 2010; Lalley 1999). The technique continued to grow in popularity over the next twenty years and has since become an established method of localized drug delivery in the pharmacological and neurochemical fields (Herr et al 2008; Herr et al 2010; Lalley 1999; Robinson et al 2008). A more recent accomplishment was the developed of a method by Herr et al to quantify iontophoretic ejection using neutral, electroactive molecules (2008).

Iontophoresis probes are constructed from glass capillaries pulled to a sharp point (~ 1 μm in diameter) making them ideal for localized delivery to discrete regions of the intact brain. Typically, multi-barrel capillaries are used as they permit one barrel to function as a recording device while the others serve as delivery pipets. Individual barrels are filled with a neuroactive drug solution prepared in an appropriate electrolyte solution, such as sodium chloride, to ensure sufficient ion flow. Probes are implanted in brain and a current is applied to each barrel solutions causing ejection of drugs into the brain region of interest (Herr et al 2010; Lalley 1999). Polarity of current is determined

by net charge on the substance of interest. Between ejections, a retaining current of opposite charge is applied to minimize diffusive leaking. Iontophoretic delivery is governed by a combination of ion migration and electroosmosis (Herr et al 2008; Herr et al 2010). The role of electroosmosis in iontophoresis, previously very controversial, was proven to be a significant contributor to the observed drug delivery by Herr et al in 2008.

Pressure ejection, on the other hand, involves the controlled expulsion of substances using applied air pressure. James Reyniers first introduced the practice in 1933 during his studies of bacterial variation. Reyniers work involving germ-free animals led to the development of an air-pressurized ejection technique that allowed substances to be delivered using micropipettes without contamination of the animals (Lalley 1999; Lindsey & Baker 2005; Reynier 1933). Chambers and Kopac later improved Reyniers' technique in 1950 and included a detailed description in their book, which was published the same year (Chambers & Kopac 1950; Lalley 1999). In 1963, Krnjev and Phillis utilized pressure ejection to apply glutamate to single cerebral cortical neurons and McCaman et al (1977), Sakai et al (1979), and Palmer et al (1980) conducted further studies involving pressure ejection in the late 1970s. Their publications described pressure-ejected delivery of known volumes of substances in the CNS (Lalley 1999). Over the past few decades, pressure ejection has proven to be very effective in the investigations of the actions and effects of substances on neurons and has been readily adopted in the neurosciences for the local administration of substances to highly discrete regions of the intact brain (Hanani 1997; Lalley 1999; Moquin et al 2012). Recently, Moquin et al (2012) developed a method of preventing reagents from leaking prematurely into the site of action by inserting an air gap into the pipet barrel.

Like iontophoretic probes, pressure ejection probes are generally fabricated from a multi-barrel glass capillary pulled to a fine point with one barrel functioning as the recording device and the others as delivery pipettes. Individual pipettes are loaded with drug solution and connected to a pressure ejection system by inlet tubing. Probes are implanted in the brain and air pressure is applied to each pipet barrel driving the

solution out of the tip and into the surrounding microenvironment (Lalley 1999; Moquin et al 2012). Pressure-ejected delivery is mainly governed by forced convection (Retterer et al 2004).

While iontophoresis and pressure ejection are both highly effective for in vivo and in vitro studies involving the local delivery of reagents, each has it own advantages/disadvantages and preferred uses. Iontophoresis, for example, is commonly used for the controlled delivery of small, charged molecules while pressure ejection is favored for delivery of uncharged or poorly charged substances as well as large molecules whose low mobility negatively affects electrically-influenced ejection (Hanani 1997; Herr et al 2010; Lalley 1999). Additionally, both methods are capable of small volume deliveries thereby minimizing tissue damage associated with the introduction of large volumes of fluid in the brain (Curtis & Nastuk 1964). However, iontophoresis offers better control over the small volume deliveries while pressure ejection requires lower concentrations of reagent (Hanani 1997; Lalley 1999). One drawback specific to pressure ejection that should be mentioned is the occurrence of an ejection-related percussive injury to brain tissue. To combat this problem, pressure ejection pipettes are generally prepared with larger tip diameters than those of iontophoresis probes (Hanani 1997). Conversely, iontophoresis suffers from pH and electroosmosis artifacts that are not observed with pressure-ejected delivery (Lalley 1999).

Both techniques face the challenge of reproducibility. Pronounced variability in the absolute amount of reagent ejected between barrels is often observed despite the maintenance of experimental conditions. This irregularity is largely attributed to differences in the inherent characteristics of the delivery pipettes arising from the materials, fabrication process, and history of use (Herr et al 2010; Lalley 1999). Ejection irregularity is typically greater in iontophoresis than pressure ejection due to changes in the electroosmotic flow (Herr et al 2010; Palmer 1980).

Another major disadvantage of these two techniques is their non-quantitative nature. Several factors contribute to the difficulty in determining the amount of drug delivered during ejection including the variability in ejections within / between barrels, changes in electroosmotic flow (iontophoresis only), and dilution from exchange of environmental media and solution in the tip (Herr et al 2010; Lalley 1999). Numerous approaches and adaptations have been made to enable quantification over the years using fluorescence, radioactivity, and electrochemistry (Herr et al 2008).

Coupling micropipettes to carbon fiber microelectrodes is beneficial to both in vivo local drug delivery techniques. It allows real-time monitoring of ejections via FSCV eliminating confounds from faulty ejections and dosage differences and simplifying analysis. In addition, it removes the need for the double penetration of the brain minimizing the destruction of brain tissue, permits the optimization of the recording site without affecting the precision of the delivery, ensures delivery of reagent to the recording site, and reduces the distance between the delivery and recording site minimizing the volume of reagent required (Herr et al 2008; Herr et al 2010; Moquin et al 2012; Wang et al 2010).

Monitoring the ejections with FSCV, however, is limited to electroactive substances. Additionally, proximity of the micropipette to the CFE can result in a diffusive leaking of reagents into the recording site, which may inadvertently affect the electrochemical response (Moquin et al 2012). This drawback can be overcome in iontophoresis by the application of a retaining current or use of high resistance pipettes controls. This control, however, is limited to charged species and has been shown to cause variability in ejection the over time (Herr et al 2010; Lally 1999). In pressure ejection, unintentional delivery is prevented by employment of an air gap in the pipet barrel; a method recently developed in our lab by Keith Moquin based on the principle of segmented flow (Moquin et al 2012). The air gap separates a vehicle solution in the tip from a reagent solution in the barrel and compresses upon the application of pressure permitting delivery. The method prevents premature drug delivery but limits the use of

each device to a single application. The continued diffusional exchange at the tip also results in increased baseline noise thereby raising the detection limit of the electrochemical reading. Furthermore, dilution of the reagent solution by the tip solution as well as difficulty controlling the volume of both the air gap and tip solution lend to the additional uncertainty in the amount of drug delivered (Moquin et al 2012).

1.2 DOPAMINE NEUROTRANSMISSION

Dopamine is derived from L-tyrosine in the cytosol of dopaminergic neuron terminals and stored in presynaptic vesicles by a vesicular membrane transporter (VMAT2) where it awaits release into the neuronal synapse (Michael & Borland 2007; Venton & Wightman 2003; Zhang et al 2007). Depolarization of the presynaptic terminal by an action potential opens voltage-gated Ca^{2+} channels triggering the fusion of storage vesicles to cell membranes and the exocytotic release of dopamine into the extracellular synaptic cleft (Borland & Michael 2004; Michael & Borland 2007; Venton & Wightman 2003; Zhang et al 2007). Cell firing and extracellular dopamine release is regulated by dopamine D2 autoreceptors (D2R). Besides autoinhibition, synaptic dopamine is also mediated by the dopamine transporter (DAT) and metabolized by catechol-o-methyl transferase. (Benoit-Marand et al 2001; Borland & Michael 2004; Herr et al 2010; Moquin et al 2009; Moquin & Michael 2011; Venton & Wightman 2003; Zhang et al 2007). DAT re-uptake removes dopamine from the extracellular space where it can then be repackaged by VMAT2 into vesicles or metabolized by monoamine oxidase (Robinson et al 2008; Venton & Wightman 2003; Zhang et al 2007).

Over the past five decades, numerous studies of dopaminergic systems have been conducted using a variety of analytical methods (Borland & Michael 2004; Clapp-Lilly et al 1999; Jaquins-Gerstl & Michael 2009; Jaquins-Gerstl et al 2011; Mitala et al

2008; Moquin & Michael 2009; Moquin et al 2012; Wang et al 2010; Wang & Michael 2012). Of these, microdialysis and voltammetry are the most widely employed for the measurement of extracellular dopamine in the brain (Borland & Michael 2004; Wang et al 2010).

Microdialysis, a well-establish sampling technique in the field of neuroscience, relies on the diffusion of small molecules across a semi-permeable membrane implanted in brain tissue. Chemical species collected in the dialysate (fluid in the membrane) are removed from the brain and analyzed externally by a wide variety of separation and detection methods such as high-pressure liquid chromatography, capillary zone electrophoresis, and mass spectroscopy (Kerh 2007; Robinson et al 2008). The power of microdialysis lies in this method of analysis, which allows the simultaneous determination of multiple chemical species, wide chemical versatility, and excellent selectivity. In addition, microdialysis is highly compatible with a multitude of animal models making it a highly popular technique for in vivo sampling of small molecules like dopamine (Jaquins-Gerstl et al 2011; Kerh 2007; Robinson et al 2008; Wang & Michael 2012).

Even so, microdialysis suffers from three major drawbacks: (1) low flow rates required for sufficient recovery and sensitivity resulting in long experimental timescales that limit temporal resolution and make measurement of sub-second events, such as dopamine release and uptake, impossible (Mitala et al 2008; Robinson et al 2008), (2) large probe dimensions (~ 150 – 200 μm in diameter, 3 – 4 mm in length) limit spatial resolution as well as result in the sampling of a sizeable portion of brain tissue thereby rendering all microdialysis measurements an average of their surroundings and masking the heterogeneity of dopamine terminals, and (3) probe implantation injuries of the surrounding brain tissue that significantly disrupts dopamine activity resulting in the measurement of tissue with an abnormal morphology and physiology (Borland & Michael 2004; Borland et al 2005; Clapp-Lilly et al 1999; Jaquins-Gerstl & Michael 2009; Kerh 2007; Mitala et al 2008; Wang & Michael 2012).

FSCV at a CFE is the alternative to microdialysis and the preferred electrochemical method for monitoring extracellular dopamine. FSCV is primarily used to monitor extracellular dopamine dynamics during electrical stimulation of the medial forebrain bundle (MFB). Unlike microdialysis, chemical analysis via FSCV occurs in vivo at the surface of the implanted microelectrode. Application of a triangular potential waveform to the CFE at a high scan rate causes electrochemically active substances, like dopamine, to oxidize (or reduce) on the electrode surface thereby generating a current response (Figure 1, Bath et al 2000; Robinson et al 2008).

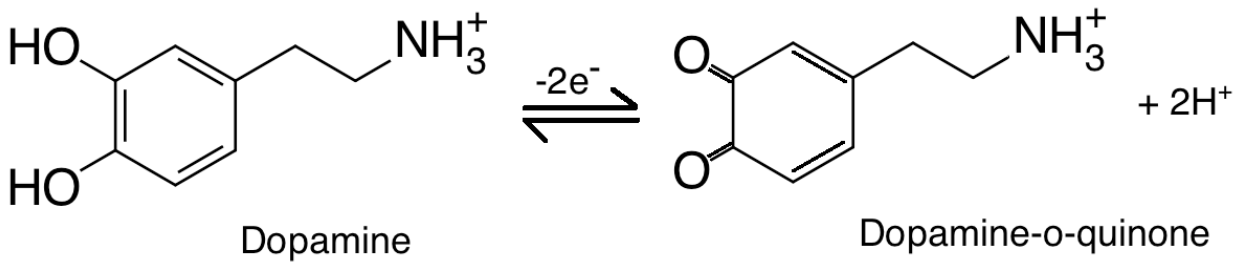

Figure 1. Dopamine redox reaction.

The power of FSCV lies in the use of high scan rates and single-digit micrometer-sized electrodes, which provide superior spatiotemporal resolution, sub-second detection, and less diffusional distortion. As a result, FSCV unlike microdialysis is capable of monitoring dynamic dopamine kinetics (Benoit-Marand et al 2001; Heien et al 2005; Moquin & Michael 2009). In addition, small probe dimensions (~ 5 – 10 μm diameter, ~ 250 – 800 μm length) minimize implantation damage and permit placement within micrometers of neuronal terminals thus making it possible to monitor local dopaminergic activity in small populations of dopamine terminals (Peters et al 2004; Jaquins-Gerstl & Michael 2009). Another major advantage of FSCV lies in the direct

proportionality between the amplitude of the dopamine current and the concentration of dopamine found at the detection site, which permits simple quantitative measurement (Bath et al 2000; Robinson et al 2008).

Nevertheless, as with microdialysis, FSCV has its limitations. For example, its chemical selectivity is limited and the electrochemical nature of the technique allows only electroactive chemicals to be measured. Furthermore, the use of high scan rates results in the generation of a large, albeit stable, background-current that must then be digitally subtracted. Identification chemical species is accomplished through recognition of characteristic reduction / oxidation peaks in background-subtracted voltammograms (Bath et al 2000; Robinson et al 2008).

1.3 OVERVIEW

The aim of this study is to establish a methodology for the local delivery of electroinactive reagents compatible with in vivo electrochemical experiments currently performed in the Michael lab. Coupling a micropipette to a CFE allows pressure-ejected delivery of electroactive compounds to be monitored and confirmed by FSCV. However, direct electrochemical monitoring of non-electroactive substances, such as raclopride, is not possible. Therefore, a method of monitoring the ejection progress of electroinactive species is needed. Here, the approach of Moquin et al (2012) is modified for use with non-electroactive reagents by the incorporation of dye into the pipet barrel. The dye is kept separated from the barrel solution by the use of a second air gap. Application of pressure pulses to the pipet barrel results in the compression of the air gaps and simultaneous mixing of the subsequent solutions. The addition of the dye allows progress of the ejections to be visually monitored. Testing of the method was performed in beaker as well as in the striatum of anesthetized rats. Ejections were monitored with

FSCV in conjunction with CFE in order to confirm functionality of the pressure ejection method. FSCV results were consistent with those found in previous studies utilizing similar methods.

2.0 METHODOLOGY

2.1 ELECTRODES

Conventional CFEs (Figure 2A, Gonon et al 1981) were constructed by inserting a single carbon fiber (7 μm diameter, T650, Cytec Carbon Fibers LLC, Piedmont, SC) into a borosilicate glass capillary (0.6 mm I.D., 1.2 mm O.D., A-M Systems Inc., Sequim, WA). The capillary was pulled to a fine tip with a vertical micropipette puller (Narishige, Los Angeles, CA) and the fiber was secured in place with a low-viscosity epoxy (Spurr epoxy, Polyscience Inc., Warrington, PA). A nichrome wire (Goodfellow, Huntington, Cambridgeshire, UK) was inserted and electrical contact was established via a mercury droplet. The exposed fiber was trimmed to a 200 μm length. Note: The crack in the CFE resulted from the EM procedure; the electrodes used in the following experiments were not cracked.

Double-barrel microelectrodes (DBE, Figure 2B) were constructed in a similar manner. Single carbon fibers were inserted into one or both sides of a double-barreled borosilicate glass capillary (0.6 mm I.D., 1.2 mm O.D., A-M Systems Inc., Sequim, WA) that was then pulled to a fine tip using a vertical micropipette puller (Kation Scientific, Minneapolis, MN). One fiber was secured in place with Spurr epoxy while the other (if present) was carefully removed creating a micropipette with a 7 μm inner diameter. Electrode fabrication was completed via the same procedure illustrated above.

All electrodes were chemically pretreated by soaking them in reagent-grade isopropanol containing activating carbon (Fisher Scientific, Fair Lawn, NJ) for 20 minutes (Bath et al 2000) and electrochemically pretreated by the application of a triangular waveform consisting of three linear potential sweeps to +1.2 V, -0.4 V, and

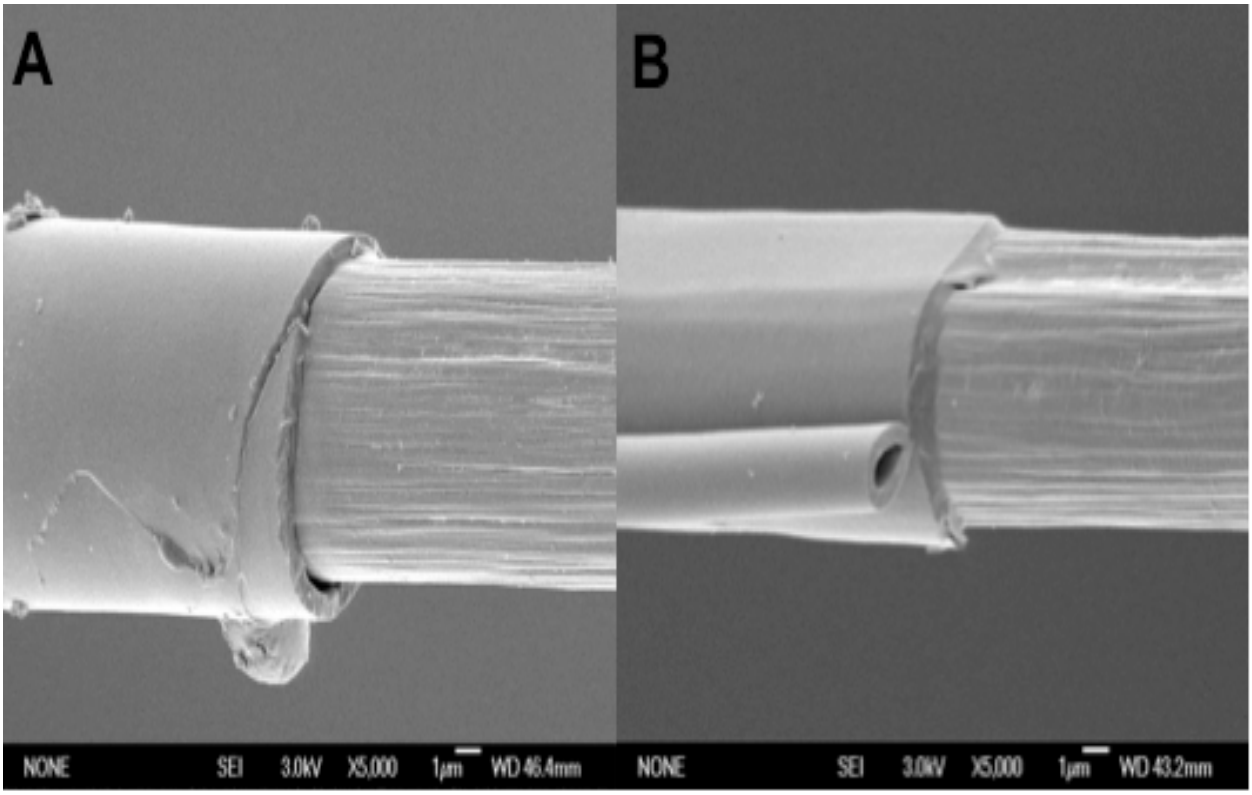

Figure 2. SEM images of (A) conventional CFE and (B) DBE.

back to 0 V prior to their use in vivo. All electrodes were post-calibrated in dopamine standards dissolved in aCSF and the results were used to convert the voltammetric responses recorded in vivo to units of dopamine concentration. The tips of the electrodes were examined by scanning electron microscopy (SEM, 5000x) and optical microscopy (500x).

2.2 EXPERIMENTAL DESIGN

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received, unless otherwise noted. Solutions of dopamine (25 μM and 30 μM) and raclopride (2 mM) were prepared by dissolving dopamine hydrochloride and raclopride tartrate separately in aCSF (1.2 mM Ca^{2+}, 152 mM Cl^{-}, 2.7 mM K^{+}, 1.0 mM Mg^{2+}, 145 mM Na^{+}, pH 7.4). All solutions were prepared using nanopure water (Nanopure, Barnstead, Dubuque, IA). The Blue #1 FD&C dye was donated by the University of Pittsburgh undergraduate stockroom.

FSCV was performed at a scan rate of 400 V/s using a high-speed potentiostat (EI-400, Ensman Instruments, Bloomington, IN) in conjunction with the software “CV Tarr Heel vs 4.3” (courtesy of Dr. Michael Heien, Department of Chemistry, Pennsylvania State University). The rest potential was 0 V vs. Ag/AgCl and the applied potential waveform consisted of three linear sweeps to +1 V, -0.5 V, and back to 0 V at a frequency of 10 Hz (Figure 3A). Dopamine oxidation currents were recorded between 0.5 V and 0.7 V on the first sweep of each scan. Voltammograms were obtained via background-subtraction (Figure 3B) and used for identification of dopamine in vivo (Figure 3C, Borland & Michael 2004).

All experiments involving the local delivery of reagents to the recording site were performed using DBEs in conjunction with a pressure ejection system. A Picospritzer III

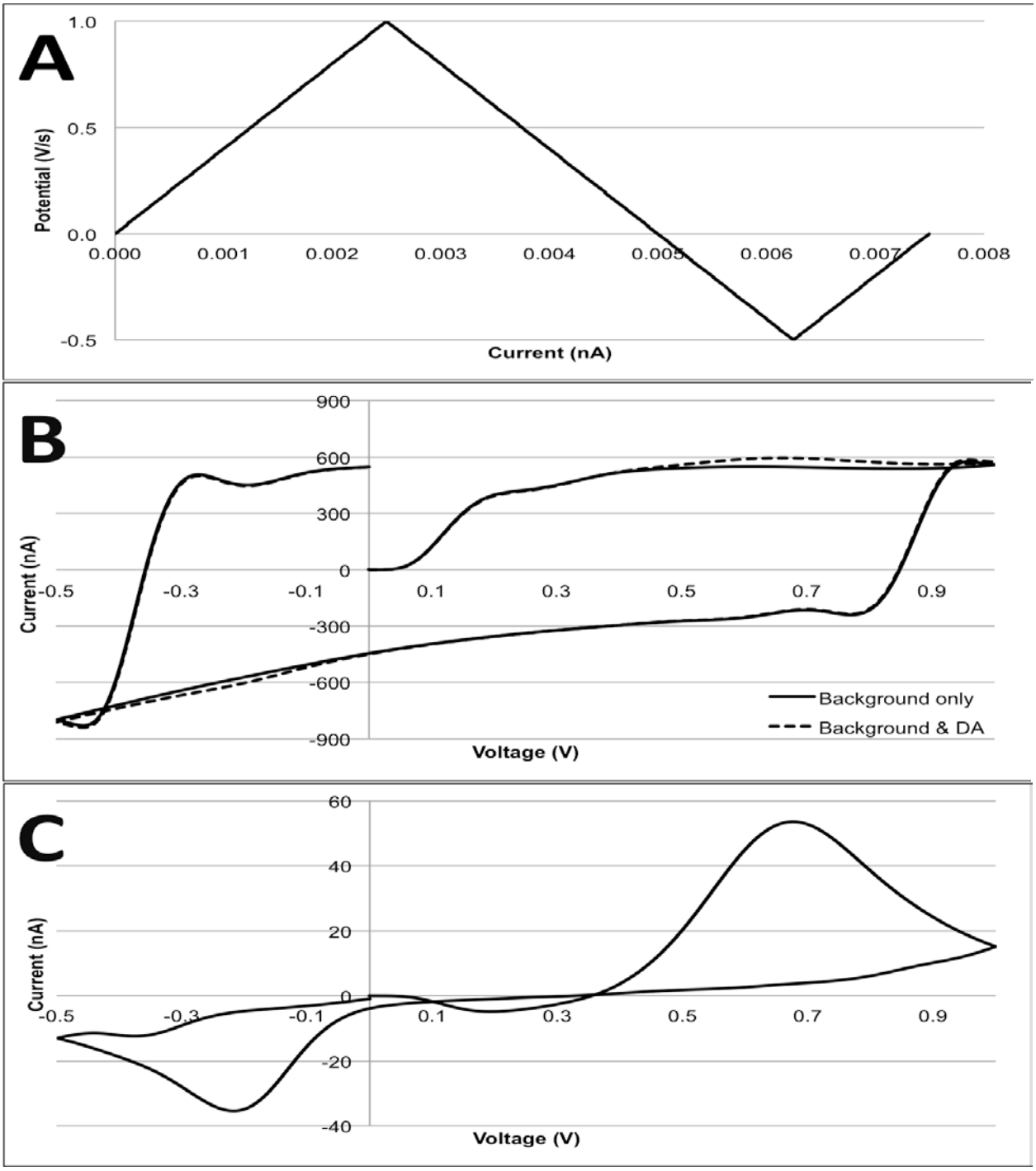

Figure 3. Diagram of a typical FSCV experiment.

(Parker Hannifin, Fairfield, NJ) was connected to the inlet end of the DBE micropipette and used to apply controlled pressure pulses to the pipet barrel. The micropipette tip was filled with aCSF vehicle and the barrel was back-filled with reagent and blue dye. Each solution was separated by an air gap of approximately a millimeter to prevent unintentional mixing (Moquin et al 2012). Ejection of the solution was accomplished by the application of pressure pulses (20 – 80 psi N_2 ; 0.2 – 5 s) to the barrel of the micropipette (Figure 4).

2.3 EXPERIMENTATION

Beaker characterization studies of the pressure ejecting were conducted using DBEs with tip diameters of ~ 7 μm unless other wise noted. The studies involved pressure-ejecting dopamine and blue dye into beakers of aCSF or agarose gel while monitoring the response with FSCV and/or a stereomicroscope.

All experiments involving animals were carried out with the approval of the Institutional Animal Care and Use Committee of the University of Pittsburgh. Male Sprague-Dawley rats (Hilltop, Scottsdale, PA) between the weights of 250 g and 350 g were anesthetized with 5% isoflurane (Butler Schein Animal Health, Dublin, OH) by volume and placed in the flat skull position (Paxinos & Watson 2005) in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Throughout each experiment, the rat's unconscious state and body temperature (37^{o}C) were maintained using 2.5% isoflurane and a homeopathic blanket (Harvard Apparatus, Holliston, MA), respectively.

Small holes were drilled through the skull and the dura was removed to allow the implantation of the working, stimulating, and reference electrodes with minimal disruption of any surrounding blood vessels (Figure 5). The Ag/AgCl reference electrode was electrically connected to the brain tissue via a salt bridge composed of a plastic

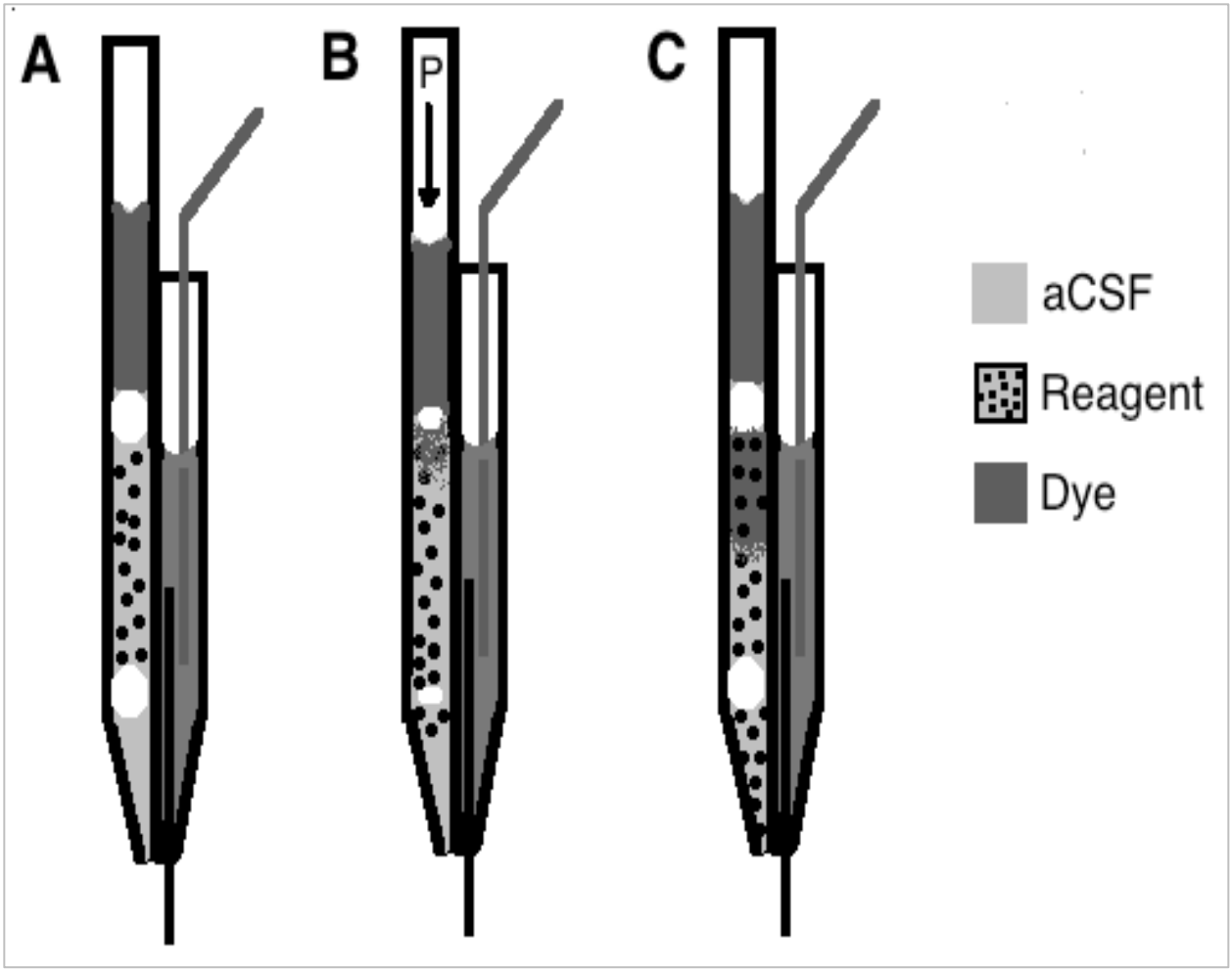

Figure 4. Cartoon schematic of a DBE utilizing segmented flow.

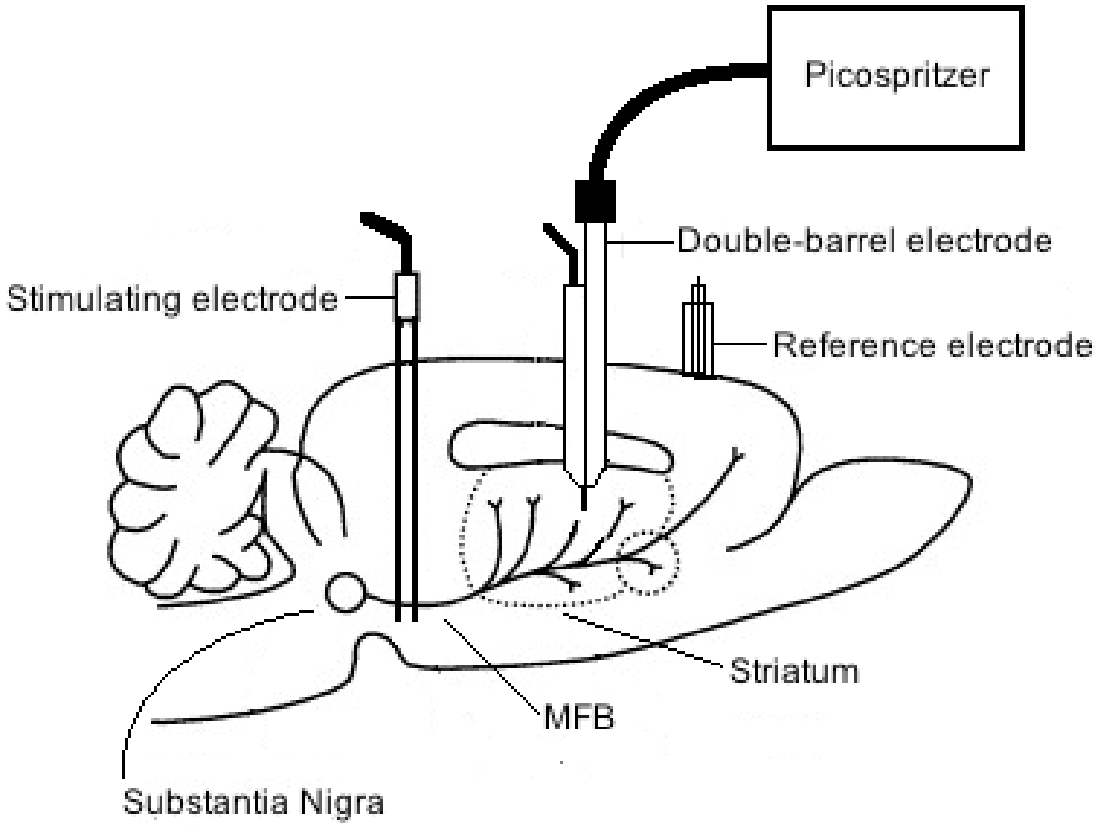

Figure 5. Cartoon diagram of in vivo experiments with DBE.

pipette plugged with tissue paper. The DBEs were implanted in the ipsilateral striatum at the following coordinates from bregma: 2.7 mm lateral, 0.7 mm posterior, and 5.0 mm below dura while the CFEs were implanted at a 10^{o} angle, 2.7 mm lateral and 0.7 mm posterior from bregma and 5.0 mm below dura (a new DBE was used with each rat). An untwisted, bipolar, stainless steel stimulating electrode was inserted above the MFB (from bregma: 1.2 mm lateral, 4.3 mm posterior, and 7.2 mm below dura). The MFB was located by lowering the stimulating electrode in small steps until an evoked dopamine response was observed. This is a well-established protocol for stimulating ascending dopaminergic fibers (Heien et al 2005). The stimulus used to evoke dopamine response in vivo was an optically isolated, constant-current, biphasic waveform with a frequency of 60 Hz, pulse height of 250 nA, pulse width of 2 ms, and duration of 3 s. Baseline signal was recorded 20 minutes after implantation.

In vivo studies of pressure ejections were conducted using DBEs with tip diameters of ~ 7 μm and conventional CFEs. Empty and aCSF containing DBEs were implanted in the striatum and monitored with FSCV. Preliminary studies involving the pressure-ejected administration of 2 mM raclopride in the striatum were also conducted.

2.4 DATA ANALYSIS

The dopamine response preceding the pressure ejection and the response recorded five minutes after completion of ejection were designated as the pre-drug and post-drug responses. For all in vivo experiments involving raclopride, t = 0 was defined as the stimulus response recorded fifteen minutes prior to the applied pressure ejection. The experimental results were averaged and statistically analyzed by one-way ANOVA.

3.0 RESULTS

3.1 CONSTRUCTION OF THE DBE

The DBEs were initially constructed by loading a single carbon fiber into one barrel of the double-barreled glass capillary and pulling the assembly to a fine point using a vertical puller. The puller settings were used to control the diameter of the pipet tips and fine-tuned as necessary. Optical images of the resulting DBEs at 500x magnification show one barrel was pulled tightly around the carbon fiber while the other remained open forming a micropipette (Figure 6). Figures 6A - 6C were prepared at the same time as were Figures 6C - 6E. Although this method provided adequate results, it was later modified to the insertion of a single carbon fiber into each of the barrels.

3.2 EFFECT OF TIP DIAMETER ON EJECTION DROPLET SHAPE

Pressure ejections (20 psi for 5 s) of blue dye into beakers containing aCSF or agarose gel from DBEs with tip diameters ranging from ~ 1 μm to ~ 20 μm were qualitatively studied under a stereomicroscope. Figure 7 depicts a cartoon illustration of the typical results observed for micropipettes with different tip diameters. Figure 7A portrays a pressure ejection from a DBE with a tip diameter of ~ 1 μm, Figure 7B illustrates the shape from a DBE in the 5 μm - 7 μm range, and Figure 7C shows that of a DBE with a tip of ~ 20 μm in diameter. Exchange between the pipet tip and the surrounding solution was observed prior to ejection in both the aCSF solution and the agarose gel.

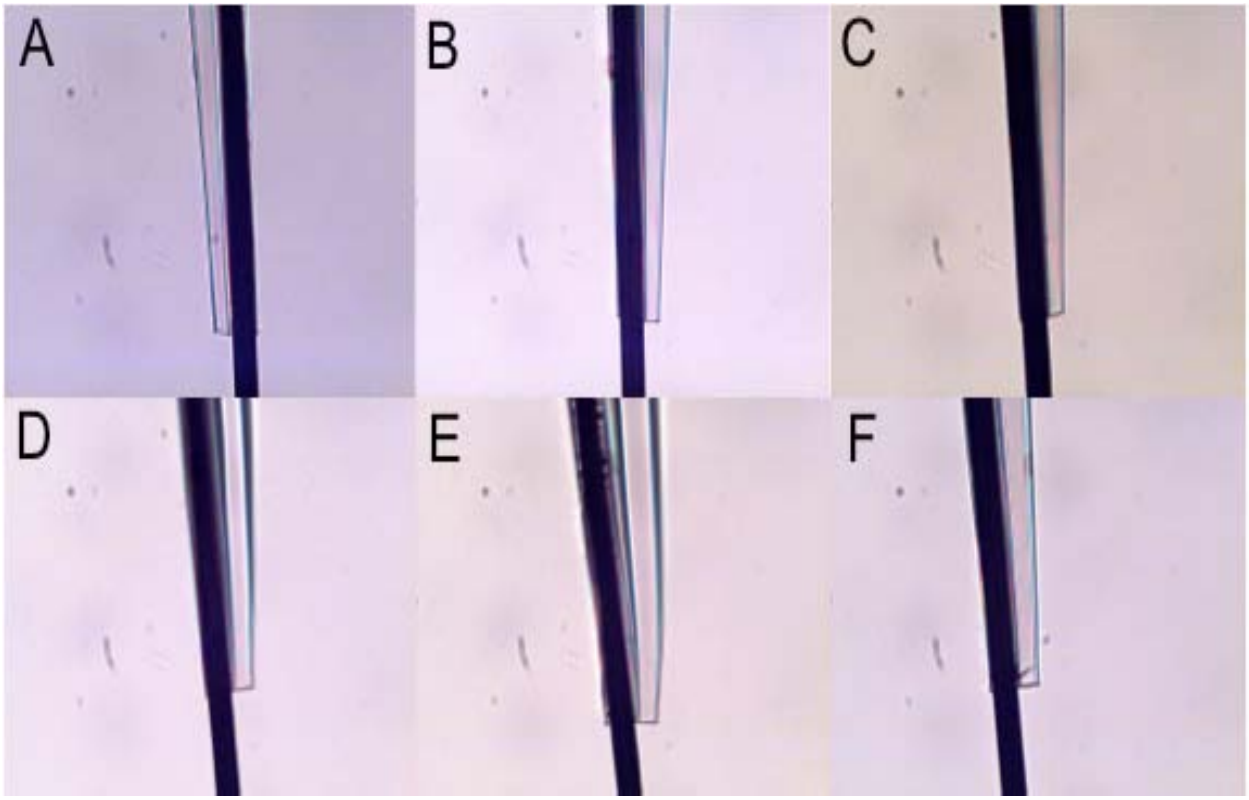

Figure 6. Images of DBEs at 500x magnification.

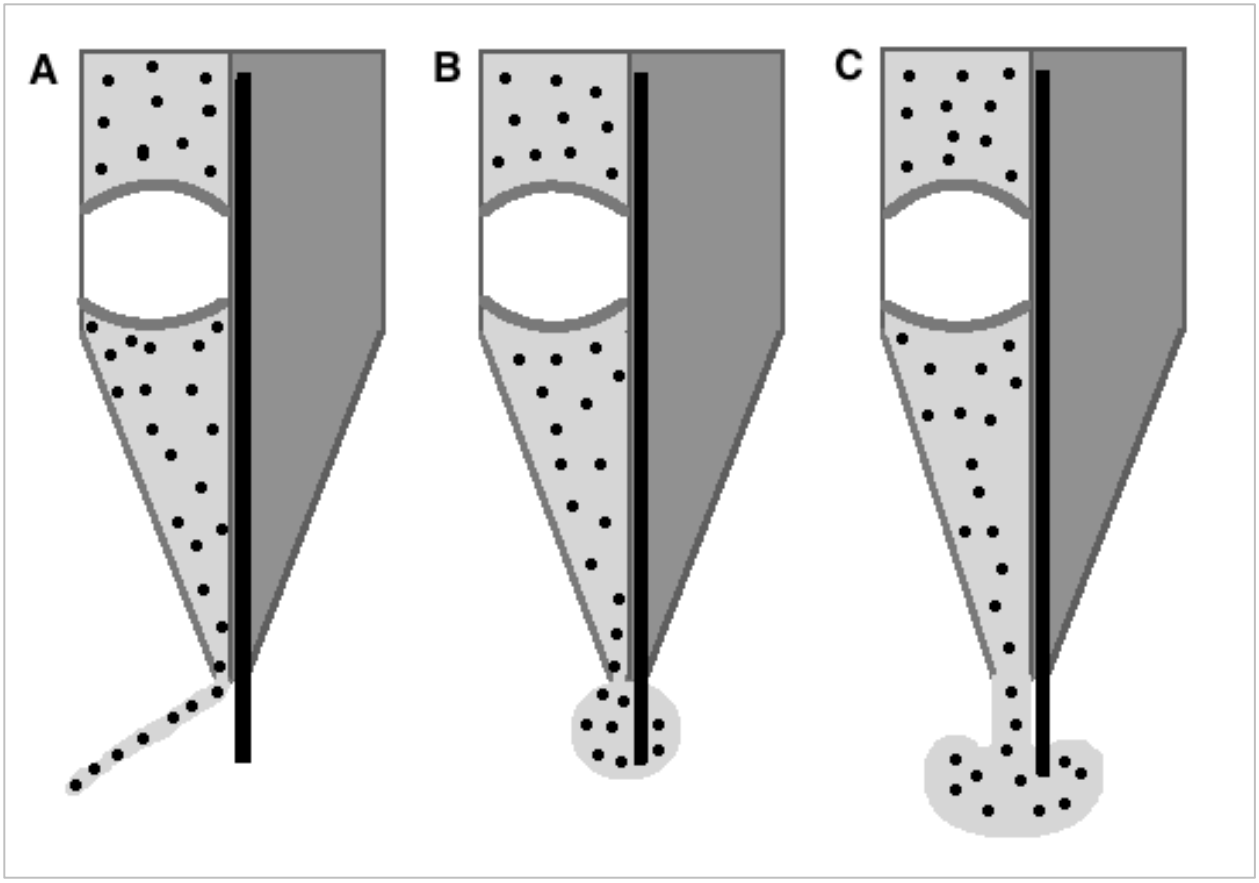

Figure 7. Schematic of pressure ejections from DBEs with varying tip diameter.

3.3 EFFECT OF PULSE PARAMETERS ON EJECTION VOLUME

DBEs containing dopamine (30 μM in aCSF) were used in the following characteristic studies and the recorded voltammetric currents were converted to units of dopamine concentration via post-calibration of the electrodes. The effect of applied pressure on the amount of reagent delivered multiple times from the same DBE is summarized in Figure 8. Figure 8A depicts the average voltammetric response observed on three separate occasions (n = 3) for a one second ejection of dopamine from a particular DBE at pressures varying from 20 – 60 psi while Figure 8B displays the average response observed on three separate occasions (n = 3) for a five second ejection of dopamine from a single DBE in the same pressure range. The average maximum dopamine concentrations of the one and five second pulses were plotted at each applied pressure with error bars depicting the standard error of the mean. The concentration of dopamine detected during ejection increased linearly with applied pressure for both duration sets (R^2 = 0.991 and 0.989).

Figure 9 shows the typical effect of pulse duration on the reagent delivery volume recorded at a single DBE (n = 1). The applied pressure was held constant at 80 psi while the pulse duration was varied from 1 – 5 s. The maximum dopamine concentration was plotted at each pulse length. The concentration of dopamine detected during ejection increased linearly with the duration of the ejection (R^2 = 0.996) as well.

3.4 VARIABILITY IN PRESSURE EJECTION

DBEs were prepared with aCSF in the tip and dopamine (25 μM in aCSF) in the barrel. Figure 10A depicts the typical voltammetric response observed for five consecutive pressure ejections (20 psi for 5 s) of dopamine from a single barrel performed five

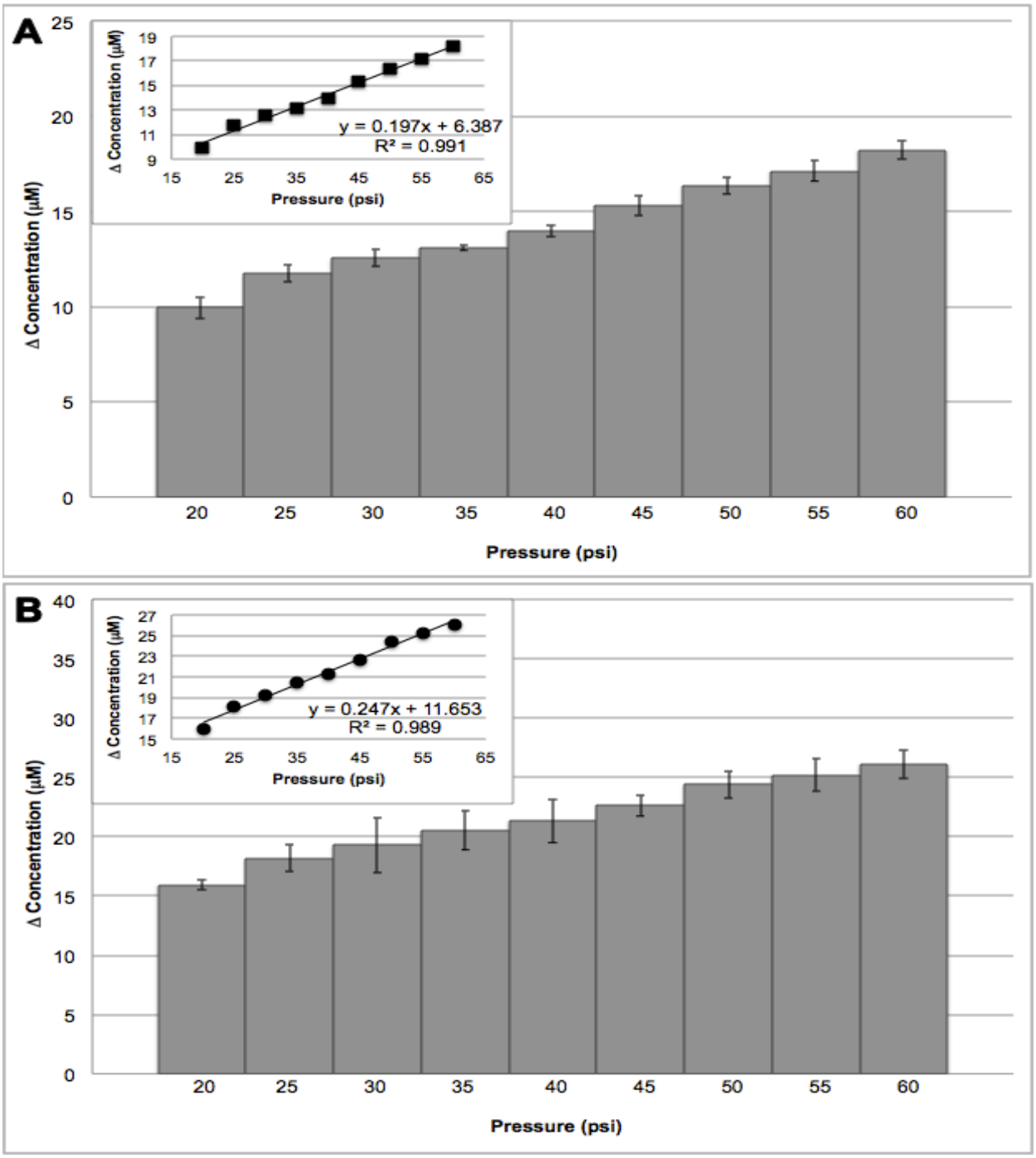

Figure 8. Pressure and ejection volume correlation for (A) 1 s and (B) 5 s ejections.

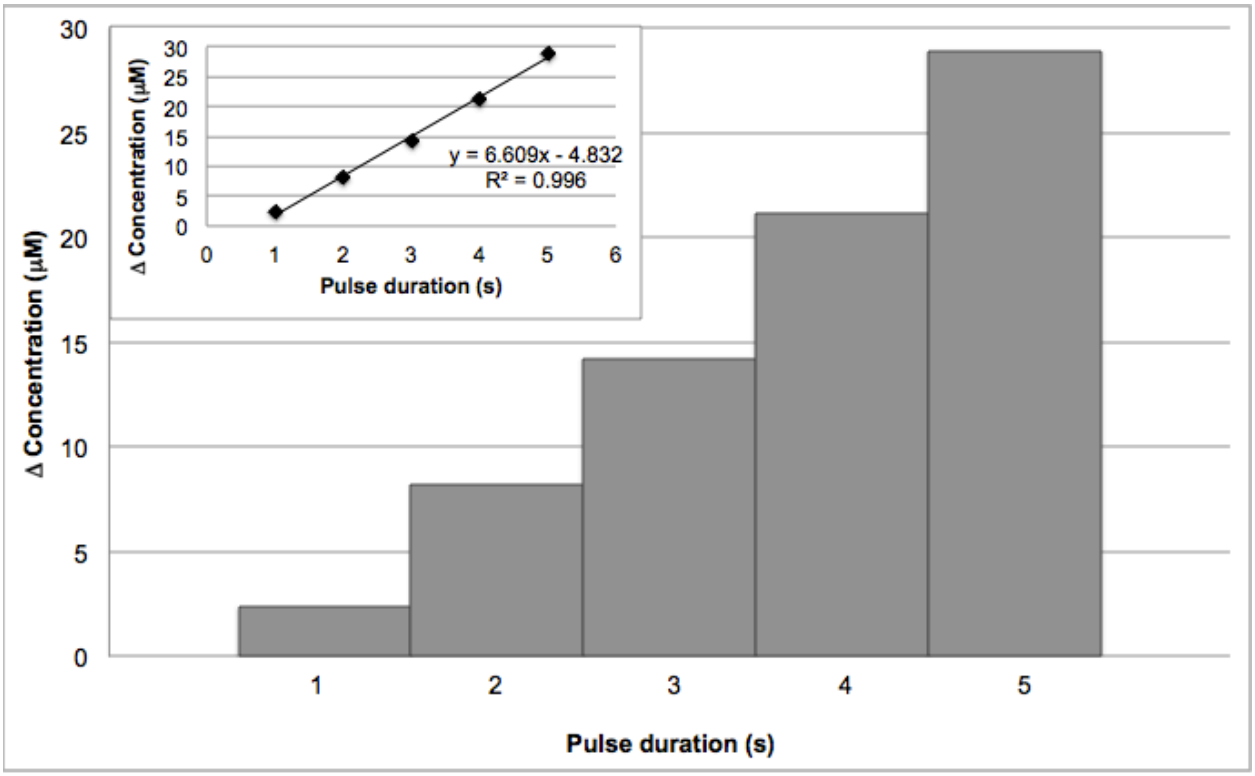

Figure 9. Correlation between pulse duration and ejection volume.

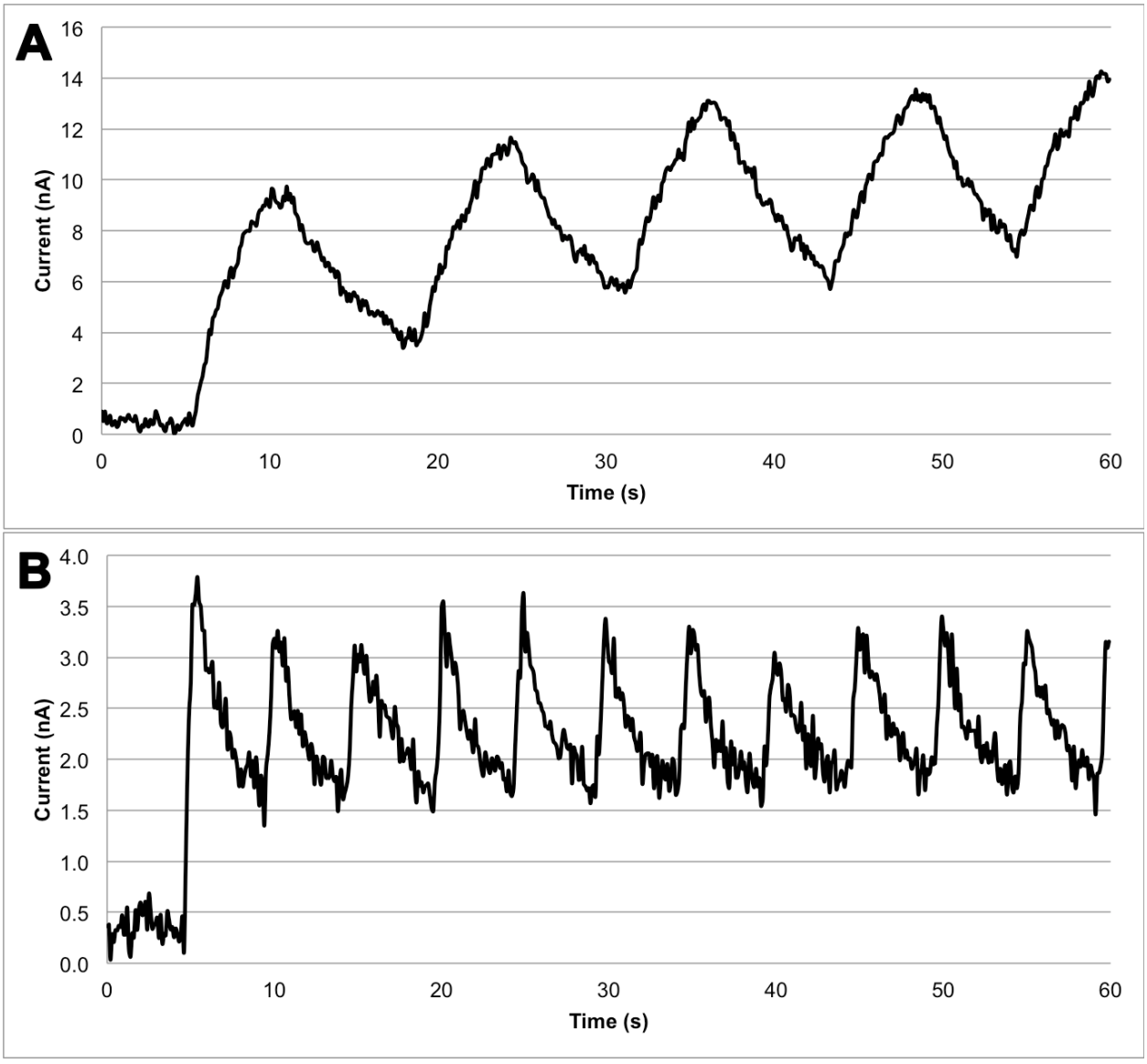

Figure 10. Consecutive pressure ejections of (A) 25 μM dopamine and (B) aCSF.

seconds apart recorded for 60 seconds. Priming pulses (20 psi for 0.2 s) were applied prior to the recording until a detectable dopamine signal was obtained. The current response exhibited a continual increase in amplitude with each additional pressure ejection. The average ejection-induced change in voltammetric current was 7.40 nA ± 0.08 nA (mean ± SEM, n = 5) and was determined by re-zeroing the start of each ejection to account for the voltammetric response not returning to the baseline.

DBEs prepared with aCSF in both the tip and the barrel were implanted in the ipsilateral striatum. Figure 10B portrays the voltammetric response observed for twelve consecutive pressure ejections (20 psi for 0.2 s) of aCSF from the same barrel in the striatum performed five seconds apart recorded for 60 seconds. The current response was fairly similar for each subsequent pressure ejection with an average ejection-induced change of 3.23 nA ± 0.06 nA (mean ± SEM, n = 12). Background-subtracted voltammograms obtained during the pressure ejection showed that the signal increase was not attributable to dopamine.

3.5 VOLTAMMETRY IN THE STRIATUM: PRESSURE EJECTION

In the following studies, DBEs containing aCSF in both the tip and barrel were implanted in the ipsilateral striatum of each rat. Stimulus responses were recorded 20 minutes after implantation every 5 minutes for a time period of 35 minutes. Note: the timing of the ejections was not automated. Background-subtracted voltammograms corresponding to the maxima of the dopamine oxidation peaks were consistent with those of dopamine so the current response was converted to units of dopamine concentration via post-calibration of the electrodes. Histograms depicting the maximum dopamine concentrations at each time point in the absence of a pressure ejection at a single DBE, in a typical rat, and in the presence of a one second pressure-ejection

(20psi) of aCSF at five separate DBEs (n=5), in five individual rats, are shown in Figure 11. A gradual decrease in the dopamine signal was observed in all rats over the course of both experiments. The amplitude of the signal loss diminished gradually with the maximum drop in dopamine signal (~ 1.6 μM and ~ 1.7 μM) occurring between t = 0 and t = 5.

Figure 12 compares the voltammetric current response obtained before and after the pressure-ejected (20 psi for 1 s) administration of aCSF recorded at five separate DBEs (n = 5), in five individual rats. After the pressure ejection of aCSF, a decrease in the voltammetric signal was observed. Background-subtracted voltammograms obtained after the aCSF pressure ejection resembled dopamine. The signal decrease in the voltammetric current, in terms of dopamine concentration, was 0.7 μM ± 0.3 μM (mean ± SEM, n = 5). The decrease in signal did not reach significance according to one-way ANOVA analysis ($f = 0.462$, $df = 1,8$).

3.6 VOLTAMMETRY IN THE STRIATUM: CONTROLS

Conventional CFEs were implanted in the ipsilateral striatum and used to record the stimulus responses 20 minutes after implantation every 10 minutes for a time period of 2 hours. The features of the background-subtracted voltammograms matched those of dopamine and thus the current responses were converted to units of dopamine concentration. A histogram depicting the average dopamine concentrations recorded at four separate conventional CFEs (n = 4), in four individual rats, is shown in Figure 13A. A gradual decrease in the dopamine signal was observed in all rats over the course of the experiment. The amplitude of the signal drop diminished over time eventually stabilizing approximately an hour and half after implantation.

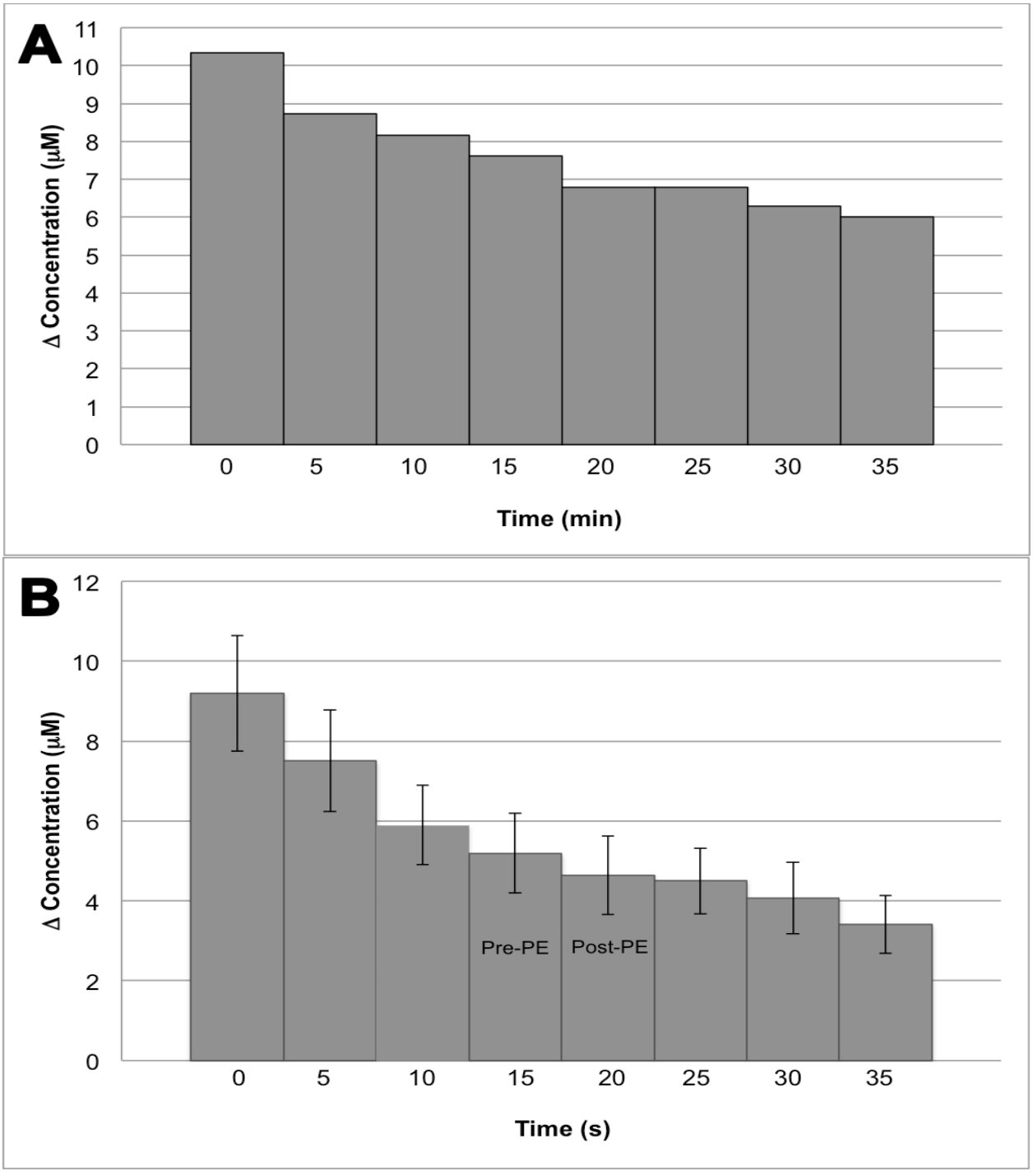

Figure 11. Evoked response in (A) absence and (B) presence of a pressure ejection.

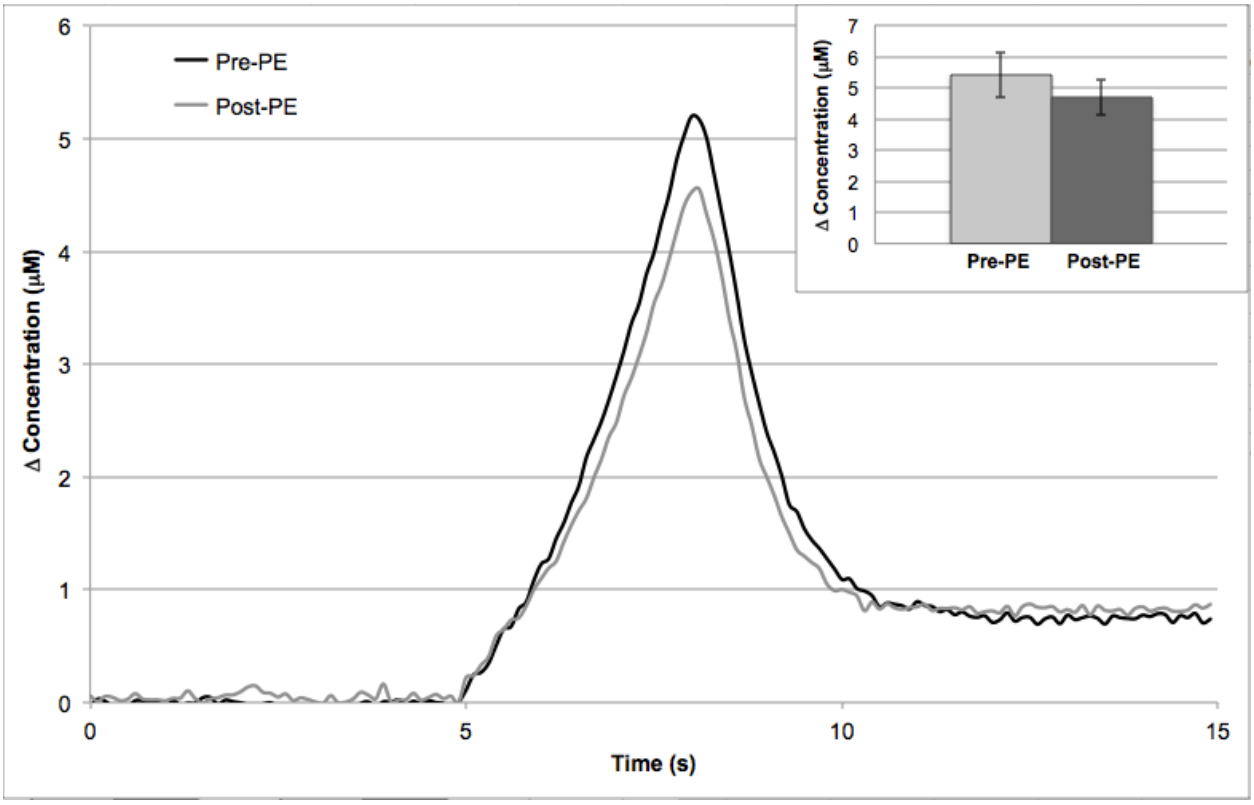

Figure 12. Effect of a pressure ejection of aCSF on evoked dopamine response.

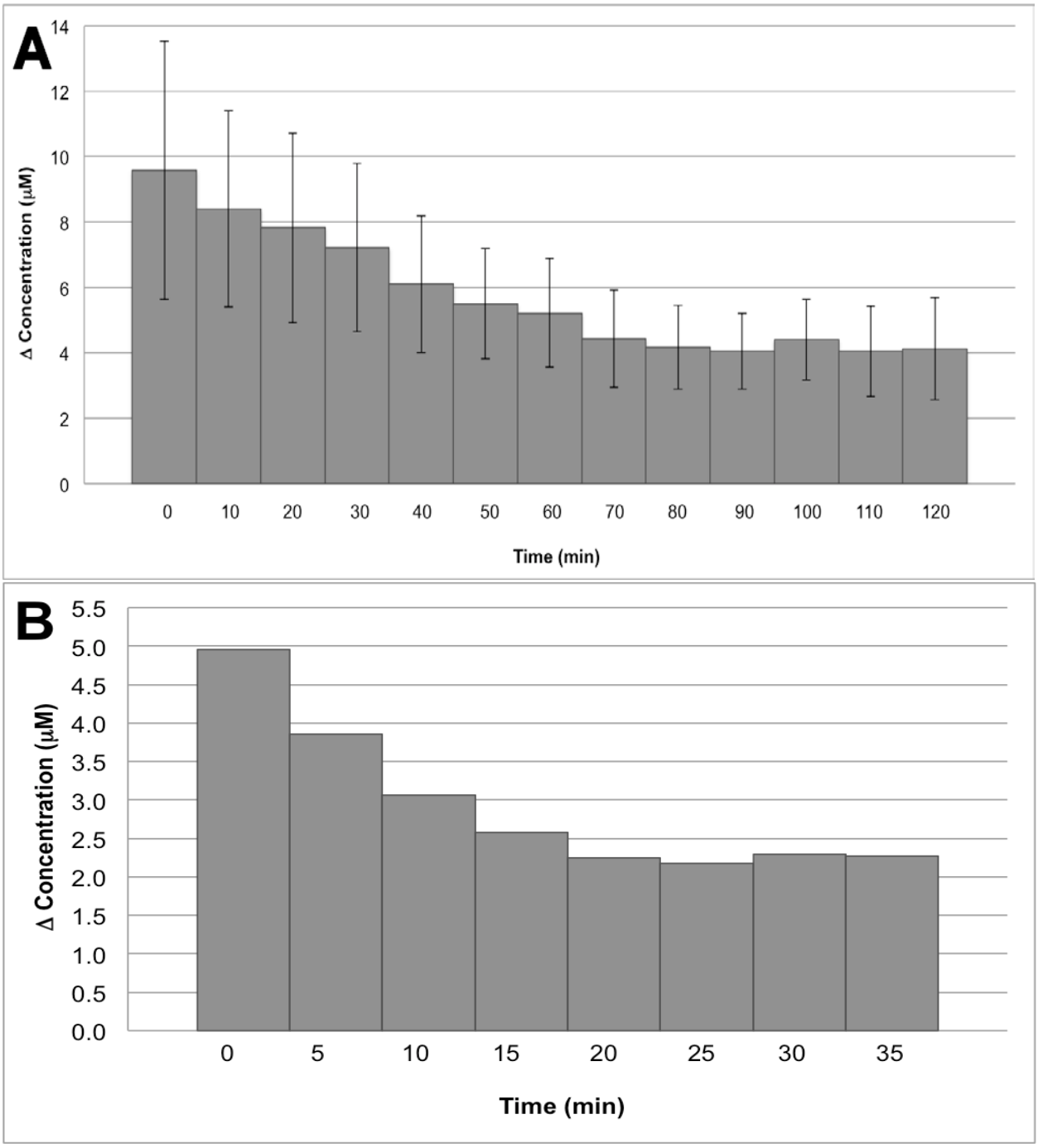

Figure 13. Dopamine response for a (A) conventional and (B) empty DBE.

Empty DBEs were implanted in the ipsilateral striatum and used to record the stimulus responses 20 minutes after implantation every 5 minutes over a 35-minute time period. As before, background-subtracted voltammograms were used to confirm the identity of dopamine and the current responses were converted to units of dopamine concentration. A histogram showing the dopamine response observed at a single DBE (n = 1) in a typical rat is shown in Figure 13B. A similar progressive loss in the dopamine signal was observed over the course of the experiment. The amplitude of the decrease diminished gradually over time with the maximum drop of ~ 1.1 μM occurring between t = 0 and t = 5.

Figure 14 depicts the maximum dopamine concentration at t = 10 and t = 15 for the above DBE experiments after normalizing the data with respect to the amplitude of the response at t = 10 in each group. A similar loss of dopamine signal was observed for each data set. The maximum signal decrease was 0.11 μM for the empty DBE, 0.12 μM for the DBE containing aCSF, and 0.11 μM for the aCSF pressure ejection.

3.7 EFFECT OF RACLOPRIDE ON THE EVOKED RESPONSE

In this study, DBEs prepared with aCSF in the tip and raclopride (2 mM in aCSF) in the barrel were implanted in the striatum. Twenty minutes after implantation, the DBEs were lowered to a naïve site and an initial stimulus response was immediately recorded. Subsequent stimulus responses were recorded every 5 minutes for a time period of 35 minutes. Priming pulses (20 psi for 0.2s) were applied at t = 10 until a non-specific voltammetric response (Figure 15) was detected followed by a one second pressure ejection (20 psi) of raclopride. Figure 16 compares the voltammetric current response obtained before and after the pressure ejection (20 psi for 1 s) of raclopride recorded at

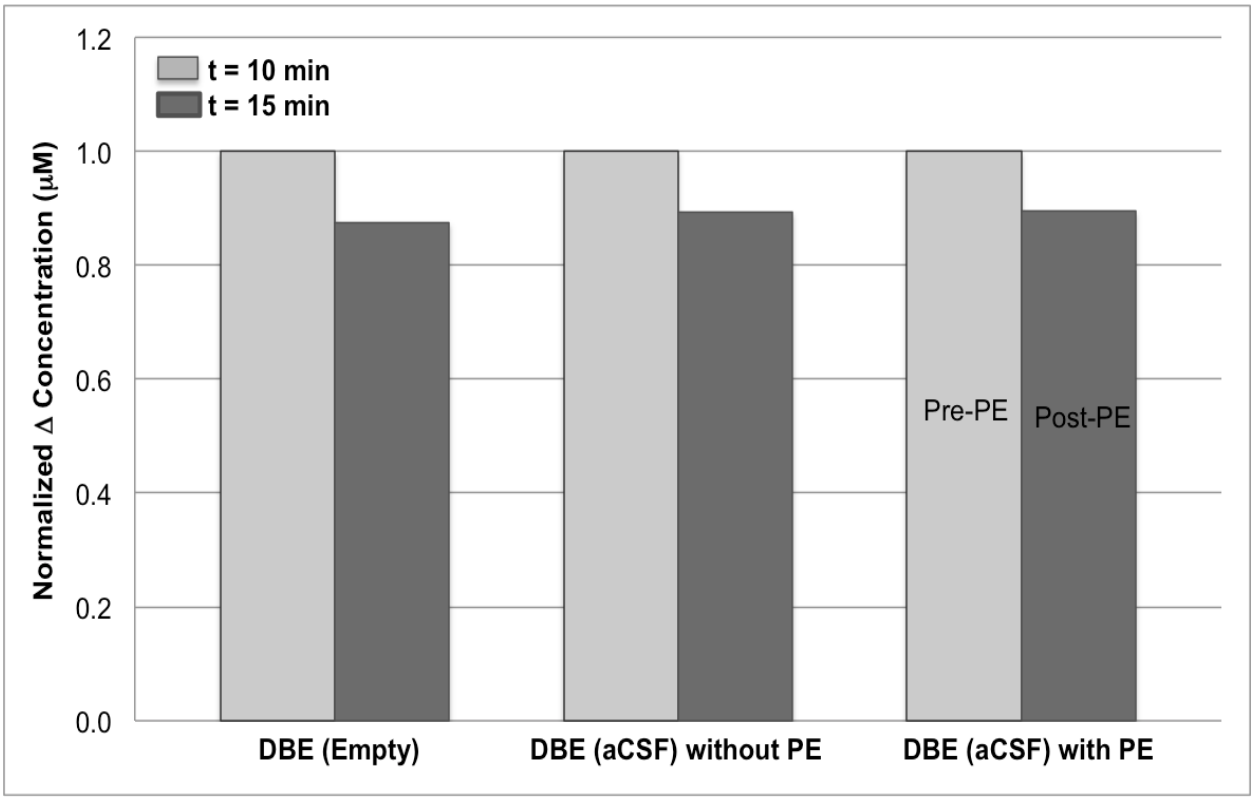

Figure 14. Normalized effect of a pressure ejection of aCSF.

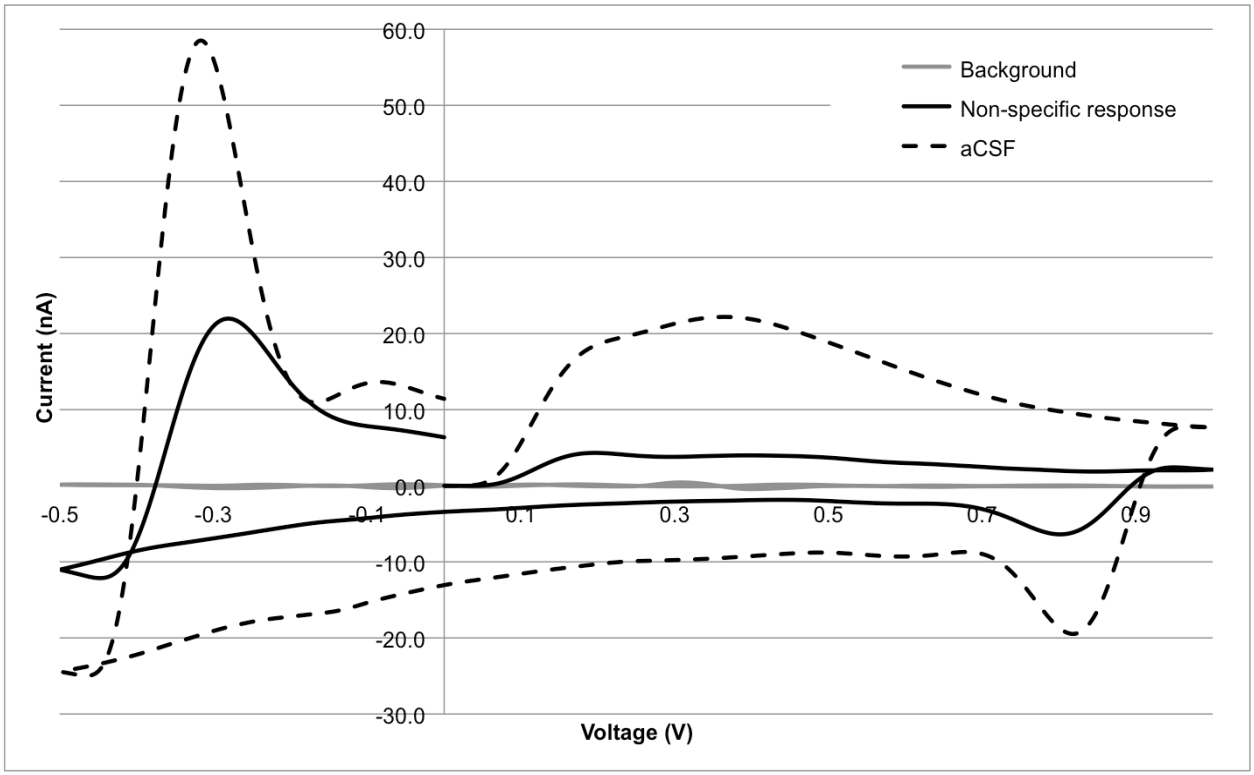

Figure 15. Non-specific raclopride response.

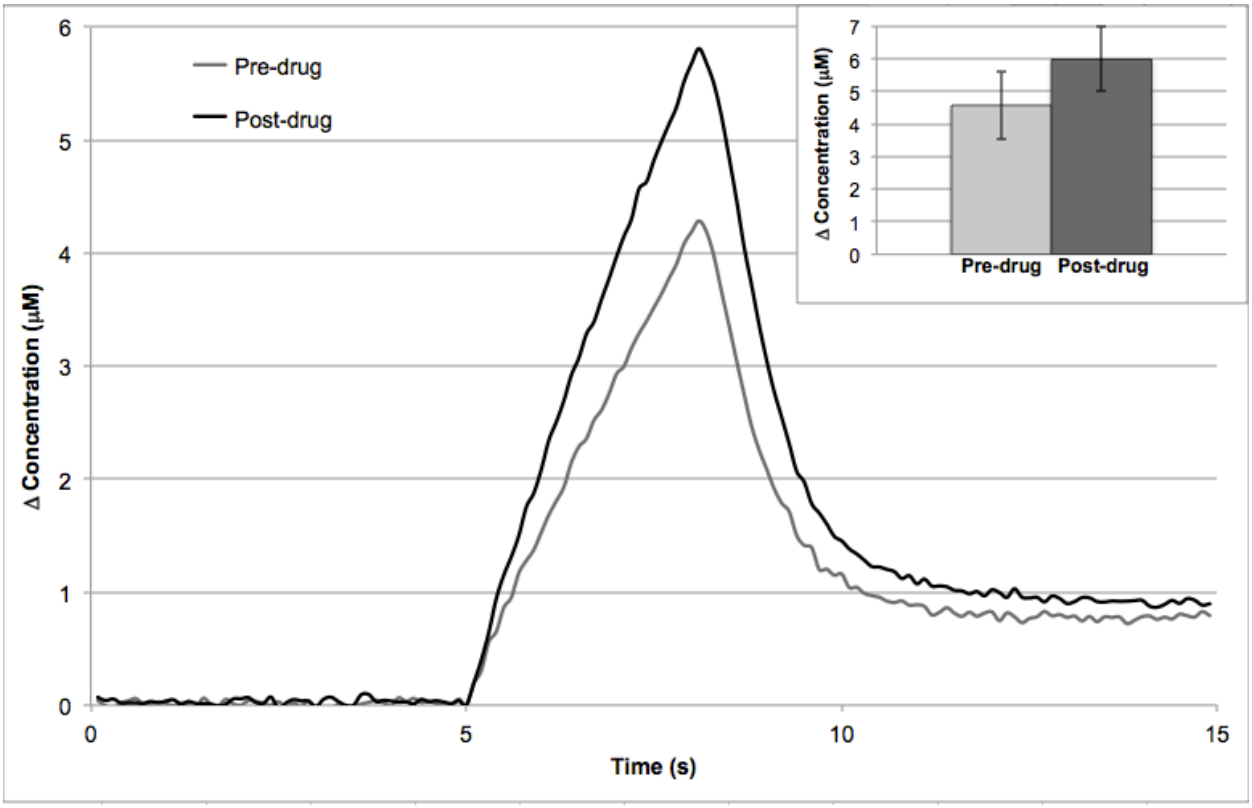

Figure 16. Effect of a pressure ejection of raclopride on evoked response.

five separate DBEs (n = 5), in five individual rats. After the pressure-ejected administration of raclopride, an increase in voltammetric signal was observed. Background-subtracted voltammograms obtained after raclopride administration resembled dopamine, therefore, the current responses were converted to units of dopamine concentration. The increase in signal after raclopride was 1.4 μM ± 1.1 μM (mean ± SEM, n = 5). One-way ANOVA analysis of the results revealed the increase did not reach significance ($f = 0.771$, $df = 1,8$).

4.0 DISCUSSION

The ability to perform quality, non-disruptive local ejections is beneficial for investigation of the neurochemistry in the CNS. DBE pressure ejection permits rapid, selective, and local delivering of reagents to discrete regions of the intact brain. Moquin et al (2012) established that the number of priming pulses required varied greatly between devices due to the inability to control volume of air gap and tip solution. Thus requiring the continuous application of priming pulses until detection of an electrochemical response. Although this is effective for employing electroactive materials, direct confirmed ejection of non-electroactive substances by electrochemical means is impossible. Incorporation of dye in the barrel allowed the progress of ejection to be monitored visually without interference with electrochemical measurements.

Previous work has shown characteristics of micropipette tips strongly influence the volume and shape of reagent delivery in addition to the ejection parameters of pulse duration and pressure (Hanani 1997; Moquin et al 2012). Fabrication of microelectrodes using two carbon fibers was observed to produce DBEs with improved reproducibility in tip diameter between devices. As a result, pressure ejections from individual DBEs as well as those between barrels were shown to demonstrated an increased consistency. Additionally, beaker experiments of pressure ejection show the DBEs maintained linearity of the ejected volume with respect to pulse duration and to applied pressure, as measured by the change in the voltammetric response of dopamine during the ejection of a dopamine solution.

In vivo validation of pressure ejections was performed in the rat striatum, a well-studied system in our lab. Proper placement was verified by the presence of stimulated DA release. During the course of the experiments it became apparent that the method suffered from a severe signal instability that continued for approximately two hours after

implantation. Previous work has shown conventional CFE equilibration and stabilization typically occurs within twenty minutes of implantation in brain tissue (Borland & Michael 2004; Heien et al 2005; Moquin et al 2012). The instability of the dopamine response was evident in all in vivo studies and is attributed to the electrochemical pretreatment of the electrode. In future work, this treatment should be discontinued in future work as previous studies have shown chemical pretreatment with isopropanol alone is sufficient for obtaining satisfactory electrode sensitivity. The amplitude of the signal loss was fairly consistent over time and between rats. Therefore, comparative analysis of the data was permitted and used to verify that in vivo pressure ejections did not significantly affect dopamine activity.

The effectiveness of the visual method of monitoring pressure ejection progress was dye tested using raclopride in vivo. Raclopride is a non-electroactive D2 antagonist well known for its ability to increase the amplitude of the evoked DA response by blocking the presynaptic D2 receptors (Herr et al 2012; Moquin & Michael 2009; Moquin et al 2012). Delivery confirmation of raclopride was previously confirmed via a non-specific electrochemical response that results from the change in solution composition upon reagent delivery (Moquin et al 2012). This response in conjunction with the observation of an increase in amplitude of the evoked response was used to confirm functionality of the approach. By monitoring the distance travelled by the dye, the number of priming pulses required from sufficient ejection could be predicted to within 1 - 2 priming pulses.

The proposed methodology proved an effective means of fabricating and employing of DBEs in vivo for the pressure-ejected delivery of reagents. The incorporation of dye into the barrel provides a simple, effective means of monitoring ejection progress.

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